

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problems Mailbox.**

**THIS PAGE BLANK (USPTO)**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12P 19/14, C08B 37/00 // (C12P 19/14, C12R 1:01, 1:645, 1:865)</b>		A1	(11) International Publication Number: <b>WO 95/30022</b>
			(43) International Publication Date: <b>9 November 1995 (09.11.95)</b>
(21) International Application Number: <b>PCT/IB95/00265</b>		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(22) International Filing Date: <b>18 April 1995 (18.04.95)</b>			
(30) Priority Data: 941581 29 April 1994 (29.04.94) NO			
(71) Applicant (for all designated States except US): A/S BIOTEC-MACKZYMAL [NO/NO]; Strandgt 3, N-9008 Tromso (NO).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors; and			
(75) Inventors/Applicants (for US only): ENGSTAD, Rolf [NO/NO]; Brattbakken 157, N-9018 Tromso (NO). KORTNER, Finn [NO/NO]; Parkgt. 13, N-8008 Tromso (NO). ROBERTSEN, Borre [NO/NO]; Hagavn. 15B, N-9007 Tromso (NO). RORSTAD, Gunnar [NO/NO]; Ivar Aasensvei 7, N-7007 Tromso (NO).			

## (54) Title: ENZYME TREATMENT OF GLUCANS

## (57) Abstract

$\beta$ -(1-6)-Glucanase treatment of glucan from yeast cells, pure or feed grade, especially yeast from the family *Saccharomyces* and particularly *Saccharomyces cerevisiae*, provides a novel glucan product suitable for use in enhancing the stimulation of host animal immune systems. Solubilization of such yeast cell glucan is further disclosed to extend the usefulness of yeast cell glucan as an adjuvant.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## ENZYME TREATMENT OF GLUCANS

5 This invention relates to the structural modification of yeast glucans, especially but not exclusively from the family Saccharomyces, by using  $\beta$ -(1-6)-glucanase, and the use of such modified glucans in vaccine and animal feed formulations.

10 Background of the Invention

15 It is known from European Patent Application Ser. No. 91111-143.3 (Publication No. 0466031 A2) that the immune system of aquatic animals can be stimulated through the administering of an effective amount of a yeast cell wall glucan. It is further known that the effect of vaccines on such aquatic animals can be enhanced by the administering of an effective amount of such yeast cell wall glucan along with the vaccine antigens.

20 Such glucan compositions are particulate glucans such as that derived from the yeast Saccharomyces cerevisiae. Such particulate glucans are macromolecular and are comprised of a chain of glucose units linked by  $\beta$ -(1-3)- and  $\beta$ -(1-6)-linkages, said glucan being a branched  $\beta$ -(1,3)-glucan having  $\beta$ -(1,3)-linked and  $\beta$ -(1,6)-linked chains therein.

25 Such particulate glucans are provided by KS Biotec-Mackzymal under the brand "MacroGard" and are potent activators of the macrophage/monocyte cell series. Thus such particulate glucans have a profound effect on the immune system.

30 While the particulate glucan derived from Saccharomyces cerevisiae is recognized to have a variety of beneficial effects on fish and other animals, the use of the glucan in the particulate and thus insoluble form is limited.

In addition it is now believed that the presence of  $\beta$ -(1-3)-branches contribute to the desired pharmaceutical benefits to be obtained from particulate glucan.

5 Accordingly a system whereby the  $\beta$ -(1-3)-linked branches are made more readily available in the glucan would be highly desirable.

Summary of the Invention

10

In accordance with the present invention it has been discovered that by treating the particulate glucan derived from yeast organisms, especially of the family Saccharomyces, and particularly Saccharomyces cerevisiae, with a  $\beta$ -(1-6)-glucanase, there is obtained a modified particulate glucan which is characterized by its enhanced activity in effecting stimulation of the immune system.

15

Thus in one embodiment of the present invention there is provided a novel  $\beta$ -(1-3)-glucan from yeast which is characterized by its enhanced ability to stimulate the immune system of fish and other animals.

20

In another embodiment of this invention there is provided a novel process for the production of  $\beta$ -(1-3)-glucan from yeast having enhanced pharmaceutical activity.

25

In another embodiment of this invention there is provided a novel solubilized  $\beta$ -(1-3)-glucan from yeast which is useful for enhancing the activity of veterinary vaccines.

30

In still another embodiment of the present invention there is provided a novel feed grade glucan composition which is useful as an ingredient in conventional animal feeds.

35 Other embodiments and advantages of this invention will be apparent from the following specifications and claims.

Process for preparation of  $\beta$ -(1-6)-glucanase treated glucan ("MacroGard").

"MacroGard" brand glucan is derived from Saccharomyces cerevisiae as disclosed in European Application Ser. No. 91111143.3. While such glucan is known to stimulate the immune system of fish, according to a preferred embodiment of the present invention, its activity is enhanced by the treatment thereof with a  $\beta$ -(1-6)-glucanase.

10

Such glucanase treatment of the glucan is carried out by suspending the glucan particle in a buffered medium at a pH in the range of about 4 to about 8 and at a temperature in the range of from about 20 to about 50°C. Suitable buffered media are those selected from the group consisting of sodium acetate, ammonium acetate and sodium-potassium phosphate. Presently preferred buffer solutions are sodium acetate or ammonium acetate. Enzymatic degradation of the glucan is commenced by the addition of the  $\beta$ -(1-6)-glucanase to the buffered medium.

$\beta$ -(1-6)-glucanases which are suitable for the modification of yeast glucan in accordance with the present invention are those obtained from a microorganism selected from the group consisting of Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma harzianum, Rhizopus chinensis, Gibberella fujikuroi, Bacillus circulans, Mucor lilmalis, and Acinetobacter. Of these a presently preferred glucanase is that obtained from Trichoderma harzianum.

30

The amount of  $\beta$ -(1-6)-glucanase employed for treatment of the glucan is normally in the range of from 1 to 50 U per g of glucan.

35

Enzymatic degradation is terminated by heating the reaction mixture to a temperature in the range of 80 to 100°C, preferably for a time in the range of 2 to 10 min. Other ways to

stop the enzyme degradation are, e.g. by adding proteases or inhibitors to the reaction mixture.

Alternatively the enzyme may be simply removed by washing.

5 The washed particles are then resuspended in water with the addition of a bactericide such as 0.3% formalin (v/v) and stored at a temperature of about 4°C.

10 The resulting enzyme treated glucan can be characterized as a branched  $\beta$ -(1-3)-glucan with  $\beta$ -(1-3)-linked sidechains being attached by a  $\beta$ -(1-6)-linkage and being essentially free of  $\beta$ -(1-6)-linked chains. In this connection the phrase "  $\beta$ (1-6) chains" is meant to include branches of more than 1  $\beta$ (1-6)-linked glucose units. The  $\beta$ -(1-6)-glucanase enzyme  
15 cleavage ensures that most chains of more than 4  $\beta$ -(1-6)-bound glucose units are cleaved off.

20 To further enhance the utility of the glucan, it is subject to solubilization. Such solubilization treatment is generally carried out at a temperature in the range of about from 70 to 90°C for a period of from about 30 to 60 min in the presence of a solubilizing agent. A presently preferred solubilizing agent is formic acid. Following solubilization the solubilizing agent is removed and the resulting glucan is boiled in  
25 distilled water.

30 In practicing the present invention glucan can be first enzyme treated and then solubilized or conversely be solubilized and then enzyme treated.

35 In accordance with another embodiment of this invention there is provided a  $\beta$ -(1-6)-glucanase treated feed grade glucan from yeast, e.g. Saccharomyces cerevisiae. Such feed grade glucan can be obtained by first contacting the yeast cell wall with an aqueous alkaline solution under conditions to effect the extraction of proteins and lipids therefrom. Generally such extraction is carried out at a temperature in

the range from about 50 to 80°C for about 2 to 8 h. A presently preferred alkaline extraction agent is sodium hydroxide. Following extraction, the cell walls are recovered from the aqueous alkaline solution and washed to remove solubilized cell wall components therefrom. The washed yeast cell wall are then neutralized by treatment with an acid such as phosphoric acid. Thereafter the neutralized washed glucan is pasteurized and then dried.

10 Suitable enzymes for treatment of the feed grade glucan are those useful in treating the high purity glucan.

15 The enzyme treated feed grade glucan is prepared by contacting the glucan with a  $\beta$ -(1-6)-glucanase in the same manner as that employed to the enzyme treatment of the glucan particulate. The  $\beta$ -(1-6)-glucanase treated feed grade glucan of this invention is useful in animal feed formulations.

20 The following examples are presented for purposes of illustration of the invention.

## EXAMPLE 1

5 This example provides the protocol used to obtain an immuno-stimulatory glucan particle suitable for utilization in the practice of the present invention.

10 500 g of dry Saccharomyces cerevisiae was suspended in 3 l of 6 % aqueous NaOH solution. This suspension was then stirred overnight at room temperature. After stirring the suspension was centrifuged at 2000 x g for 25 min. The supernatant was discarded and the insoluble residue was then resuspended in 3 l of 3 % NaOH and incubated for 3 h at 75°C followed by cooling the suspension overnight. The suspension was then centrifuged at 2000 x g for 25 min and the supernatant was decanted. The residue was then resuspended in 3 % NaOH, heated and centrifuged as previously described.

15 20 The insoluble residue remaining was then adjusted to pH 4.5 with acetic acid. The insoluble residue was then washed with 2 l of water three times and recovered by centrifuging at 2000 x g for 25 min after each wash (the supernatant was poured off). The residue was then suspended in 3 l of a 0.5 M aqueous acetic acid. The suspension was heated for 3 h at 90°C. The suspension was then cooled to room temperature. 25 After cooling, the insoluble residue was then collected by centrifuging at 2000 x g for 25 min. This treatment (from adjusting to pH 4.5 to collecting the cooled residue) was repeated 6 times.

30 35 The insoluble residue was then suspended in 3 l of distilled water and stirred for 30 min at 100°C, then cooled and centrifuged at 2000 x g for 25 min. The supernatant was discarded. The insoluble residue was washed in this manner 4 times. The residue was next suspended in 2 l of ethanol and heated at 78°C for 2 h. This wash with ethanol was repeated 4 times. The residue was then washed 4 times with 3 l of distilled water at room temperature to remove the ethanol, thereby

providing a suspension of desired glucan product.

EXAMPLE 2

5

This example provides the protocol to obtain glucan particles essentially free of  $\beta$ -(1-6)-linked chains with the use of  $\beta$ -(1-6)-glucanase isolated from Trichoderma harzianum.

10 200 mg of glucan particles prepared in accordance with Example 1 were suspended in 40 ml 50 mM ammonium acetate buffer, pH 5.0, together with 10 U of  $\beta$ -(1-6)-glucanase at 37°C for 6h with constant stirring. The enzymatic degradation of the glucan particles was ended by heating the suspension 15 at 100°C for 5 min. The particles were then washed three times with 200 ml sterile distilled H<sub>2</sub>O by centrifugation at 2000 x g for 10 min, whereafter 185 mg of dried enzyme treated glucan was obtained.

20 The enzyme treatment will only cleave  $\beta$ -(1-6)-linkages within  $\beta$ -(1-6)-linked chains, but will not remove the  $\beta$ -(1-6)-linked glucosyl residue extending from the branching points. The resulting enzyme treated glucan can be characterized as a branched  $\beta$ -(1-3)-glucan with  $\beta$ -(1-3)-linked sidechains being 25 attached by a  $\beta$ -(1-6)-linkage and being essentially free of  $\beta$ -(1-6)-linked chains.

EXAMPLE 3

30

This example provides the protocol to solubilize glucan particles prepared in accordance with Example 1 by hydrolysis using formic acid (HCOOH).

35

2.0 g of glucan particles were suspended in 1.0 l of 90% formic acid and heated at 80°C for 45 min under constant stirring. The suspension was cooled to 35°C and the formic

acid was evaporated. The residue containing the hydrolysed particles was boiled in 500 ml distilled water for 3 h, whereafter the cooled suspension was filtrated through a 0.44  $\mu\text{m}$  filter, frozen and lyophilized whereby 1.9 g of dry solubilized particles were obtained. The lyophilized solubilized particles were then dissolved in 100 ml distilled water and dialyzed, using a tubular dialysis membrane having a nominal molecular weight cut off (NMWCO) of 5000 Dalton, against tap water for 24 h, and then lyophilized. This resulted in 1.8 g solubilized glucan product.

## EXAMPLE 4

15 This example demonstrates the biological effects of glucan particles prepared according to Example 1, and  $\beta$ -(1-6)-glucanase treated glucan particles prepared according to Example 2 on immune responses in Atlantic salmon.

20 An A-layer positive isolate of Aeromonas salmonicida subsp. salmonicida, referred to as strain no. 3175/88 (Vikan Veterinary Fish Research Station, Namsos, Norway) was used. The bacterium was grown in brain heart infusion broth (Difco, USA) for 30 h at 14°C in a shaker incubator, and the culture medium with the bacterium was centrifuged for 10 min at 3000  $\times g$ . The pellet was resuspended in 0.9% saline, and the bacterium was killed by adding 0.5% formalin (v/v) to the suspension and incubating at 14°C for 24 h. The formalinized culture was then washed with sterile 0.9% saline and resuspended to a concentration of  $2 \times 10^9 \text{ ml}^{-1}$  bacteria in 0.9% saline with 0.3% formalin. Bacterial suspensions were mixed with an equal volume of saline or with the different glucan suspensions ( $10 \text{ mg ml}^{-1}$  in saline). Formalin was added to the vaccines to a final concentration of 0.3% (v/v).

25

30

35

In carrying out these experiments, two groups of experimental fish were used. In the vaccine experiment, Atlantic salmon

presmolts of 20 - 40 g were used. In the experiment where serum was collected for measuring blood lysozyme activity after glucan injection, Atlantic salmons of 50 - 70 g were used. The fish were kept in 150 l tanks supplied with aerated fresh water at 12°C and fed with commercial pellets ad libitum twice daily.

In the vaccination experiment 40 fish in each group were IP-injected with 0.1 ml of the different vaccine preparations or vaccine without glucan as a control. Blood was collected in evacuated tubes (Venoject, Terumo-Europe, Belgium) from 10 fish in each group 6, 10, and 18 weeks after injection. Blood samples were allowed to clot overnight at 4°C and sera were collected after centrifuging the tubes at 2000 x g for 10 min. Individual serum samples were transferred to Micronic serum tubes (Flow Laboratories Ltd., Lugano, Switzerland) and stored at -80°C until required.

In order to measure the effect of glucans on blood lysozyme activity, salmons were IP injected with 0.3 ml of the different glucans in saline or with 0.3 ml saline as the negative control. The glucans were administered at a concentration of 10 mg ml<sup>-1</sup>. Blood samples were collected from 10 fish from each group 10 and 20 days after injection, using evacuated tubes (Venoject). The tubes were kept on ice until centrifuged at 2000 x g for 10 min, and individual serum samples were transferred to Micronic serum tubes and stored at -80°C until required.

Lysozyme activity was measured with the turbidimetric method using 0.2 mg ml<sup>-1</sup> lyophilized Micrococcus lysodeikticus as the substrate in 0.04 M sodium phosphate buffer at pH 5.75. Serum (20 µl) was added to 3 ml of the suspension and the reduction in absorbance at 540 nm was measured after 0.5 min and 4.5 min at 22°C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min<sup>-1</sup>. Results are expressed as mean lysozyme activity in serum from 10 fish (Tables 1 and

2).

The level of specific antibody against the A-layer of A. salmo-nicida in salmon sera was measured by an enzyme-linked immunosorbent assay (ELISA). A-layer protein was purified from whole A. salmonicida cells (Bjørnsdottir *et al.* (1992), Journal of Fish Diseases, 15:105-118), and protein content was determined (Bradford, M.M. (1976), Analytical Biochemistry, 72:248-254) using a dye-reagent concentrate from Bio-Rad Laboratories (Richmont, USA). Microtitre plates were coated with 100  $\mu$ l of 5  $\mu$ g  $ml^{-1}$  A-layer protein in 50 mM carbonate buffer, pH 9.6, and incubated overnight at 4°C. The further procedure was performed as described by Håvardstein *et al.* (Journal of Fish Diseases (1990), 13:101-111). The antibody titre in pooled serum samples was determined before individual serum samples were measured at three different dilutions (1:500, 1:1000 and 1:2000). Absorbance was read at 492 nm in a Multiscan MCC/340 MK II (Flow Laboratories Ltd). Results are expressed as mean antibody response to the A-layer of the bacterium at a dilution of 1:2000 in serum from 10 fish (Tables 1 and 2).

Table 1. Differences in biological effects of glucan particles and  $\beta$ -(1-6)-glucanase treated glucan particles on immune responses in Atlantic salmon.

5

	Saline control	Untreated glucan particles	$\beta$ -(1,6)-glucanase treated glucan particles
--	----------------	----------------------------	---

Lysozyme activity

10 post injection  
(units/ml)

10 days	304	505	529
20 days	330	407	454

15

-----

	Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with $\beta$ -(1,6)-glucanase treated glucan particles
--	------------------------	---	--

Antibody response

post injection  
(absorbance)

25 6 weeks	0.165	0.255	0.376
10 weeks	0.059	0.355	0.500
18 weeks	0.037	0.197	0.142

30

Both injection of untreated and  $\beta$ -(1,6)-glucanase treated glucan particles induced significantly higher ( $p<0.01$ ) lysozyme activity in serum compared to saline control both 10 and 20 days post injection. At day 20 post injection the lysozyme levels in fish injected with  $\beta$ -(1,6)-glucanase treated glucan particles were significantly higher ( $p<0.05$ ) compared to fish injected with untreated particles.

10  $\beta$ -(1,6)-glucanase treated glucan particles induced significantly higher ( $p<0.05$ ) antibody response to the vaccine compared to vaccine without adjuvant at all three sampling times, whereas untreated glucan particles induced significantly higher response 10 and 18 weeks post injection.  $\beta$ -(1,6)-glucanase treated glucan particles induced significantly higher ( $p<0.05$ ) antibody response than did untreated glucan particles at 10 weeks post injection, whereas no significant differences between the two were observed at 6 and 18 weeks post injection.

20

Table 2. Biological effects of glucan particles and solubilized glucan.

		Saline control	Untreated glucan particles	Solubilized glucan particles
5				
10	10 days after injection	304	505	603
20	20 days after injection	330	407	773
15	-----			
20		Vaccine without glucan	Vaccine with untreated glucan particles	Vaccine with solubilized glucan particles
25				
30	6 weeks after injection	0.165	0.255	0.184
35	10 weeks after injection	0.059	0.355	0.349
30	18 weeks after injection	0.037	0.197	0.120

Injection of solubilized glucan particles induced significant higher ( $p<0.01$ ) lysozyme activity than did untreated glucan particles both 10 and 20 days post injection. No significant differences could be observed between the ability of solubilized glucan particles and untreated glucan particles to

induce increased antibody response to the vaccine antigen at any sampling time point. Both induced significant higher ( $p<0.05$ ) antibody response than vaccine without adjuvant 10 and 18 weeks post injection, but not at 5 weeks post injection.

#### EXAMPLE 5

10 This example provides the protocol to obtain a glucan composition suitable for use in the feeding of animals.

15 1000 kg of dry cell wall material of Saccharomyces cerevisiae was suspended in 5300 l of water at a temperature of 65°C in a stainless steel tank. To the suspension of cell walls in water there was added 227 l of 50% w/w NaOH so as to provide a caustic concentration of about 3%. The resulting mixture was then stirred for a period of about 4 h at a temperature of about 60°C.

20 25 Following the initial extraction period the suspension was then diluted with 8000 kg of water at a temperature of about 65°C in a stainless steel, stirred, washing tank such that the weight of the mixture was doubled. The resulting diluted mixture was then maintained at a temperature of about 60°C while being stirred for a period of about 15 min. Thereafter the resulting mixed diluted suspension was centrifuged in a nozzle centrifuge (Alfa Laval DX209). The supernatant was discarded. The resulting concentrated cell wall suspension was continuously introduced into a second steel stirred wash tank containing 8000 kg water and the mixture adjusted by the addition of water to give a final weight of 14500 kg. The resulting suspension was then mixed for a period of 15 min at a temperature of 60-65°C. Thereafter the agitated mixture was centrifuged.

30 35 The resulting cell wall suspension was continuously added to

a third vessel containing 8000 kg water. Additional water at 60°C was added to provide a final weight of 14500 kg. The resulting suspension was stirred for a period of 15 min at 60-65°C and thereafter centrifuged.

5

Following centrifugation, the resulting cell wall concentrate was transferred to a stainless steel storage tank wherein the suspension was cooled to a temperature of about 5-10°C. The resulting cooled suspension was treated with phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) in a stainless steel agitated tank in an amount to achieve a suspension of solids having a pH of 5.5-7.5.

10 Following neutralization the resulting neutralized mixture was subjected to pasteurization by heating at a temperature of 75°C for a period of 18 seconds by passing the mixture through an in-line plate and frame heat exchanger.

15 Following pasteurization the resulting pasteurized mixture was then spray dried in a spray drier maintained at an inlet air temperature of at least 140-150°C and an exhaust temperature of about 65-70°C whereby there was achieved 300 kg of dry glucan product.

25 EXAMPLE 6

This example provides the protocol and effect of treatment of feed grade glucan with a  $\beta$ -(1-6)-glucanase.

30 25 g of feed grade glucan, prepared in accordance with Example 5, suspended in 1.25 l of 50 mM sodium acetate, pH 5.0, in a 2 l conical flask. Glucan particles were maintained in suspension by shaking, the suspension was warmed to 30°C and purified  $\beta$ -(1-6)-glucanase from Trichoderma harzianum was 35 added to a final concentration of 1.8 U/g glucan.

To follow the timecourse of the enzymatic removal of  $\beta$ -1,6-

bound glucose from the glucan particle, 1 ml aliquotes of the suspension were withdrawn at different timepoints, centrifuged at 2000 x g, and 0.2 ml of the supernatants analyzed for free, reducing carbohydrate (Nelson *et al.* (1944), 5 Journal of Biological Chemistry, 153:315-80). The glucan suspension was incubated for 28 h at which time the rate of release of free, reducing carbohydrate was observed to be very low. The glucan particles were then pelleted by centrifugation at 2000 x g, washed once in 50 mM sodium-10 acetate, pH 5.0 and once in water.

A fine, dry powder suitable for use as a feed additive was prepared from the wet glucan by first dehydrating the pellet four times with ethanol at room temperature followed by air 15 drying at room temperature.

Results from treating a feed grade glucan with  $\beta$ -(1-6)-glucanase from *T. harzianum* as described above are shown in Table 3.

Table 3. Liberation of glucose from feed grade glucan during treatment with  $\beta$ -(1-6)-glucanase from T. harzianum.

5	Enzyme reaction time, [h]	Glucose liberated, [% of total glucose in glucan]
10	0	0.0
	0.5	1.9
	1	2.6
	2	3.3
	3	3.7
	4	4.0
	5	4.3
15	2	5.5
	8	5.6

## c l a i m s

1. A process for the preparation of a glucan product from  
5 yeast which comprises:

(a) contacting a branched  $\beta$ -(1-3)-glucan having  $\beta$ -(1-3)-linked and  $\beta$ -(1-6)-linked chains therein with a  $\beta$ -(1-6)-glucanase under conditions such that the resulting glucan is  
10 comprised of  $\beta$ -(1-3)-linked glucose units and is essentially free of  $\beta$ -(1-6)-linked chains.

2. A process according to claim 1 wherein said  $\beta$ -(1-6)-glucanase is obtained from the groups of microorganisms  
15 consisting of Trichoderma longibrachiatum, Trichoderma reesei, Trichoderma harzianum, Rhizopus chinensis, Gibberella fujikuroi, Bacillus circulans, Mucor lilmalis and Acinetobacter.

20 3. A process in accordance with claim 1 wherein said  $\beta$ -(1-6)-glucanase is obtained from Trichodermia harzianum.

4. The process of claim 1 wherein the particulate  $\beta$ -(1-3)-glucan is derived from yeast of the family Saccharomyces.

25 5. The process of claim 4 wherein the particulate  $\beta$ -(1,3)-glucan is derived from Saccharomyces cerevisiae.

30 6. The process of claim 1 wherein said insoluble particulate  $\beta$ -(1-3)-glucan is prepared by the process comprising:

(a) alkali-extracting suitable glucan-containing yeast cells with a suitable extractive aqueous alkali solution under suitable conditions to provide a first insoluble yeast residue.  
35

(b) hot alkali-extracting said first insoluble yeast residue

with a suitable extractive aqueous alkali solution under suitable extraction conditions wherein the hot alkali extraction is performed at least 2 times to provide a second insoluble yeast residue and recovering the insoluble yeast residue after hot alkali extraction; thereafter

5 (c) washing said second insoluble yeast residue with a suitable hydrolyzing acid under suitable conditions with water at a pH in the range of from about pH 4 to about pH 7 thereby providing a third insoluble yeast residue and recovering said third insoluble yeast residue after the wash;

10 (d) hydrolyzing said third insoluble yeast residue under mild acidic hydrolysis condition wherein the acid hydrolysis is performed at least 3 times to provide a fourth insoluble yeast residue and recovering the yeast residue after each acid hydrolysis; thereafter

15 (e) boiling said fourth insoluble yeast residue under suitable conditions in water wherein the boiling of said fourth insoluble yeast residue is performed at least 2 times to provide a fifth insoluble yeast residue and recovering the insoluble yeast residue after each boiling; and

20 (f) boiling said fifth insoluble yeast residue under suitable conditions in ethanol wherein the boiling in ethanol of said fifth yeast residue is performed at least 2 times to provide a sixth insoluble yeast residue and recovering the insoluble yeast residue after each boiling; thereafter

25 (g) washing said sixth insoluble yeast residue under suitable conditions with water wherein the washing of said sixth yeast residue is performed at least 2 times to provide a yeast glucan and recovering the insoluble yeast residue after each wash.

7. The product of the process of claim 1, being characterized as a branched  $\beta$ -(1-3)-glucan with  $\beta$ -(1-3)-linked sidechains being attached by a  $\beta$ -(1-6)-linkage and being essentially free of  $\beta$ -(1-6)-linked chains.

5

8. The product of the process of claim 6, being characterized as a branched  $\beta$ -(1-3)-glucan with  $\beta$ -(1-3)-linked sidechains being attached by a  $\beta$ -(1-6)-linkage and being essentially free of  $\beta$ -(1-6)-linked chains.

10

9. An insoluble particulate yeast glucan especially from the yeast family Saccharomyces and particularly from the yeast species Saccharomyces cerevisiae being characterized as a branched  $\beta$ -(1-3)-glucan with  $\beta$ -(1-3)-linked sidechains being attached by a  $\beta$ -(1-6)-linkage and being essentially free of  $\beta$ -(1-6)-linked chains.

15

10. A process for the production of a solubilized  $\beta$ -(1-3)-glucan particle from yeast, especially from the yeast family Saccharomyces and particularly from the yeast species Saccharomyces cerevisiae, which comprises contacting an insoluble glucan from the yeast family Saccharomyces having a backbone of  $\beta$ -(1-3)-linked glucose units with at least one  $\beta$ -(1-3)-linked side chain of at least 1 glucose units attached thereto with a solubilizing agent.

25

11. A process in accordance to claim 10 wherein said solubilizing agent is formic acid and said insoluble glucan is contacted with said solubilizing agent at a temperature in the range of from 70 to 90°C.

30

12. The solubilized  $\beta$ -(1-3)-glucan product of the process of claim 11.

35

13. A process for the preparation of a feed glucan product from yeast, especially from the yeast family Saccharomyces and particularly from the yeast species Saccharomyces

cerevisiae, which comprises:

(a) contacting the feed grade yeast glucan being a branched  $\beta$ -(1-3)-glucan having  $\beta$ -(1-3)-linked and  $\beta$ -(1-6)-linked chains therein with a  $\beta$ -(1-6)-glucanase under conditions such that the resulting glucan is comprised of  $\beta$ -(1-3)-linked glucose units and is essentially free of  $\beta$ -(1-6)-linked chains.

10 14. The process of claim 13 wherein said glucan is derived from Saccharomyces cerevisiae.

15 15. The process of claim 14 wherein said feed grade glucan is prepared by the process comprising:

(a) contacting yeast cell walls with an aqueous alkaline solution under suitable conditions to effect the extraction of proteins and lipids therefrom;

20 (b) separating the resulting extracted yeast cell walls from said aqueous alkaline solution;

(c) washing the resulting separate yeast cells so as to further remove solubilized cell wall components therefrom;

25 (d) neutralizing the washed yeast cell walls; and

(e) pasteurizing the neutralized, washed cell walls and thereafter drying the resulting pasteurized, neutralized, washed cell walls.

30 16. The product of the process of claim 13, being characterized as a branched  $\beta$ -(1-3)-feed grade glucan with  $\beta$ -(1-3)-linked sidechains being attached by a  $\beta$ -(1-6)-linkage and being essentially free of  $\beta$ -(1-6)-linked chains.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 95/00265

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C12P19/14 C08B37/00 // (C12P19/14, C12R1:01, 1:645, 1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12P C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 028 703 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 2 July 1991 see column 2, line 48 - column 3, line 50 see claims 1-15 ---	1-6, 10-16
X	DATABASE WPI Section Ch, Week 7949 Derwent Publications Ltd., London, GB; Class B04, AN 79-88295B & JP,A,54 138 115 ( KIRIN BREWERY KK) , 26 October 1979 see abstract ---	7-9, 16 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search  21 July 1995	Date of mailing of the international search report  03.10.95
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer  Douschan, K

Form PCT/ISA/210 (second sheet) (July 1992)

## INTERNATIONAL SEARCH REPORT

Intern. al Application No

PCT/IB 95/00265

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS LETTERS, vol. 64, no. 1, 1976 pages 44-47, S.BALINT ET AL. 'Biosynthesis of Beta-Glucans catalyzed by a particulate enzyme preparation from yeast' see the whole document ---	7-9, 16
X	J. BIOCHEM., vol. 98, no. 5, 1985 pages 1301-1307, MASAO SHIOTA ET AL. 'Comparison of Beta-Glucan structures in a cell wall mutant of <i>Sacch. cerevisiae</i> and the wild type' see the whole document ---	7-9, 16
P, X	DEV. COMP. IMMUN., vol. 18, no. 5, 1994 pages 397-408, R. ENGSTAD, B. ROBERTSEN 'Specificity of a Beta-Glucan Receptor on macrophages from atlantic salmon' see the whole document ---	1-16
A	EP,A,0 466 037 (PHILLIPS PETROLEUM COMP.) 15 January 1992 cited in the application see claims 1-6 -----	1-16

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 95/00265

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5028703	02-07-91	US-A-	5250436	05-10-93
EP-A-0466037	15-01-92	AU-B-	628752	17-09-92
		AU-A-	7933891	23-01-92
		CA-A-	2040374	07-01-92
		JP-A-	4253703	09-09-92
		US-A-	5401727	28-03-95